

Progressive Regulation of Sesquiterpene Biosynthesis in *Arabidopsis* and Patchouli (*Pogostemon cablin*) by the miR156-Targeted SPL Transcription Factors

Running title: Age-Dependent Sesquiterpene Biosynthesis Regulation

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Short Summary

The miR156-SPL module directly regulates expression of sesquiterpene synthase genes. This uncovers a molecular link between developmental timing and sesquiterpene production, and suggests a new strategy to boost essential oil production in a shortened period of plant growth.

ABSTRACT

Plant metabolites vary at different stages of life cycle. Although it is well documented that environmental factors stimulate biosynthesis of secondary metabolites, the regulation by endogenous developmental cues remains poorly understood. The microRNA156 (miR156)-targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) factors function as a major age cue in regulating developmental phase transition and flowering. We show here that the miR156-targeted SPL transcription factor plays an important role in the spatiotemporal regulation of sesquiterpene biosynthesis. In *Arabidopsis thaliana*, the miR156-SPL module regulates the formation of (*E*)- β -caryophyllene in flowering stage through modulating expression of the sesquiterpene synthase gene *TPS21*. We demonstrated that SPL9 directly binds to *TPS21* promoter and activates its expression. In the perennial fragrant herb *Pogostemon cablin*, the accumulation of “patchouli oil”, largely composed of sesquiterpenes dominated by (-)-patchoulol, is also age-regulated, and the SPL promotes biosynthesis of sesquiterpenes in elder plants by up-regulating patchoulol synthase (*PatPTS*) gene expression. As miR156-SPLs are highly conserved in plants, our finding not only uncovers a molecular link between developmental timing and sesquiterpene production, but also suggests a new strategy to engineer plant for accelerated growth with enhanced production of terpenoids.

Keywords: miR156 targeted SPLs, *TPS21*, *PatPTS*, (*E*)- β -caryophyllene, patchoulol, sesquiterpene biosynthesis, *Pogostemon cablin*, *Arabidopsis*.

INTRODUCTION

Plants produce copious low molecular weight organic compounds known as specialized or secondary metabolites, of which terpenoids form the largest and most diversified group (Pichersky and Gang, 2000; Falara and Pichersky, 2012). During sesquiterpene biosynthesis, the C5 isoprene units of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are produced by the cytosolic mevalonate (MVA), then prenyltransferases catalyze the condensation of DMAPP with IPP to form farnesyl diphosphate (FPP), which are converted by terpene synthases (TPSs) into sesquiterpenes (C15), most of them are cyclic. They can be further modified by such reactions as oxidation, dehydrogenation and acylation, etc., resulting in the great diversity of sesquiterpenoid products (Bohlmann et al., 1998; Newman and Chappell, 1999; Dudareva et al., 2004). TPSs are of central importance because they not only direct the isoprenoid flux into specific groups of metabolites, but also determine the basic skeletons of the products, thus the final terpenome (Christianson, 2008; Degenhardt et al., 2009; Tholl and Lee, 2011).

Plant terpenoids have many physiological and ecological functions, such as serving as phytoalexins in defense against pests and pathogens, and signals in bio-communications and interactions, thus they contribute greatly to plant survival and successful reproduction in natural ecosystem (Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002; Engelberth et al., 2004; Maffei et al., 2011). In addition, plant terpenoids also possess a great commercial value, as some terpenoids are widely used as flavorings, fragrances and pharmaceuticals, which have stimulated many attempts to increase their yield in plant (Goff and Klee, 2006; Li and Vederas, 2009). Patchouli (*Pogostemon cablin*), a perennial herbaceous species of the Mint family (Lamiaceae or Labiatae), is a commercial fragrant plant that produces patchouli oil, which is distinct for primarily consisting of sesquiterpenes, with (-)-patchoulol as the dominant constituent (Deguerry et al., 2006). Five sesquiterpene synthases from patchouli have been identified, of which four converted FPP into one major product, respectively, with PatTpsA for γ -curcumene, PatTpsCF2 for

(+)-germacrene A, and both PatTpsBF2 and PatTpsB15 for (-)-germacrene D. The last and also the pivotal one, patchoulol synthase (PatPTS, PatTps177), is a multi-product sesquiterpene synthase responsible for the production of the vast components of patchouli oil, including patchoulol and at least 13 other sesquiterpenes (Deguerry et al., 2006).

Mounting investigations have been focused on the regulation of terpenoid biosynthesis related to stress signals. However, production and accumulation of secondary metabolites in plant are often organ- or tissue-specific, and their amounts and compositions change during plant development and aging (Facchini and De Luca, 1995; Gershenzon et al., 2000). For examples, in peppermint (*Mentha × piperita* L.) monoterpenes are present in peltate glandular trichomes instead of capitate ones, and they accumulate rapidly for the first 21 days of early leaf development, then level off and sustain at a relatively stable level during the rest of leaf life (Gershenzon et al., 2000; Turner et al., 2000). In snapdragon (*Antirrhinum majus*) the components of floral scent, including myrcene, ocimene and methylbenzoate, are primarily released from the upper and lower petal lobes of the flower, and the emissions increase rapidly on the second day after anthesis and reach the maximum at the sixth day followed by declining thereafter (Dudareva et al., 2003). These reports imply that the biosynthesis of terpenoids in plant is subjected to developmental stage regulation.

A. thaliana has a similar feature of terpene biosynthesis and emission. After flowering a blend of terpenes are released from the inflorescence, with (*E*)- β -caryophyllene as the predominant constituent (Aharoni et al., 2003; Chen et al., 2003). There are 32 *TPS* genes in *Arabidopsis* genome (Aubourg et al., 2002), among them two sesquiterpene synthases, *TPS21* and *TPS11*, are responsible for the vast floral sesquiterpenes (Tholl et al., 2005). *TPS21* catalyzes the conversion of FPP into (*E*)- β -caryophyllene plus minor amounts of α -humulene and α -copaene, and *TPS11* is responsible for the formation of essentially all of the remaining floral sesquiterpenes including (+)-thujopsene (Aharoni et al., 2003; Chen et al., 2003; Tholl et al., 2005). Both *TPS11* and *TPS21* genes are predominantly expressed in flower with *TPS11* in

nectaries and ovules whereas *TPS21* highly in stigma and moderately in sepals and anther filaments (Chen et al., 2003; Tholl et al., 2005). It has been reported that the AUXIN RESPONSE FACTOR 6 (ARF6) and ARF8 trigger the expression of two JA inducible genes, *MYB21* and *MYB24*, by enhancing JA production, which in turn promote petal, stamen, gynoecium and nectary development and consequently affect the sesquiterpene production (Mandaokar et al., 2006; Reeves et al., 2012), indicating that biosynthesis of sesquiterpenes in flowers is related to floral organ development and flower maturation. Nonetheless, to date a direct molecular link between plant development and terpene biosynthesis is lacking.

In plant miR156, an evolutionary highly conserved microRNA (miRNA), plays a key regulatory role in both vegetative phase transition and flowering through targeting a subset of transcription factors named SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) (Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009; Huijser and Schmid, 2011). In addition, SPLs participate in controlling fertility (Xing et al., 2010; Xing et al., 2013), embryo development (Nordine and Bartel, 2010), cell size (Wang et al., 2008; Usami et al., 2009), leaf initiation rate (Schwarz et al., 2008; Wang et al., 2008) and trichome distribution (Shikata et al., 2009; Yu et al., 2010). Besides these developmental aspects, the miR156-targeted SPLs are also connected to the biosynthesis of phenylpropanoids, as SPL9 was shown to repress anthocyanin accumulation by destabilizing the MYB-bHLH-WD40 transcriptional activation complex (Gou et al., 2011).

Serving as an endogenous age cue for timing plant development, miR156 levels off gradually after germination, leading to increased levels of the targeted SPLs during plant growth and development (Axtell and Bartel, 2005; Wang et al., 2009; Wu et al., 2009). Here, we report that the progressive biosynthesis of (*E*)- β -caryophyllene in *Arabidopsis* inflorescence after flowering is regulated by miR156-targeted SPLs, which directly activate *TPS21* expression. We further show that in the perennial herb patchouli a similar regulatory mechanism is functioning: the SPL regulates both patchouli oil production and vegetative phase transition.

RESULTS

Arabidopsis TPS21 Expression Changes with miR156 Level

After flowering, *Arabidopsis* inflorescences emit a range of volatiles, of which 60% are terpenes with sesquiterpenes taking the great majority (Aharoni et al., 2003; Chen et al., 2003). Several *TPS* genes, including *TPS03*, *TPS14*, and *TPS24*, as well as the two well characterized sesquiterpene synthase genes of *TPS11* and *TPS21*, were found to be highly or preferentially expressed in flowers (Chen et al., 2003; Hong et al., 2012). To further examine their temporal expression patterns, we collected the inflorescences at different days after flowering (DAF) and conducted quantitative real-time PCR (qRT-PCR). We found that, in general, transcript levels of the five *TPS* genes were increasing in developing flowers (Figure 1A-1E). In particular, *TPS21* exhibited the most striking enhancement of expression, reaching the maximum level at 18 DAF (Figure 1A-1E). Because *TPS11* and *TPS21* are responsible for most of sesquiterpenes, which take 95% of *Arabidopsis* floral terpene volatiles, we chose them for further investigation. Consistent with previous results, gas chromatography-mass spectrum (GC-MS) analysis showed that the floral emissions of *TPS21* products, (*E*)- β -caryophyllene and α -humulene, were elevating after flowering (Figure 1F). The increased release was also observed for (+)-thujopsene (Figure 1F), though to a less extent.

Since miR156-targeted SPLs function in promoting the vegetative-to-reproductive transition and flowering, we asked whether they were involved in regulating terpene biosynthesis in inflorescence. First, we detected the volatiles from inflorescences of transgenic plants over-expressing miR156 (*Pro35S:MIR156*), or a mimicry form of miR156 (*Pro35S:MIM156*) which elevates the level of SPLs (Franco-Zorrilla et al., 2007). The amount of (*E*)- β -caryophyllene, a dominant sesquiterpene component, was substantially increased in *Pro35S:MIM156* but drastically reduced in *Pro35S:MIR156* floral emissions, compared to those of the wild-type (Figure 2A and Supplementary Figure 1 online), indicating that miR156 indeed played a role in regulating terpenes biosynthesis. The amount of

(+)-thujopsene released, however, remained to be low in either the *Pro35S:MIM156* or the *Pro35S:MIR156* emissions (Figure 2A).

To uncover how miR156 controls *Arabidopsis* sesquiterpene biosynthesis, we analyzed the expression of genes of the sesquiterpene biosynthetic pathway. In plant cell, FPP, a common precursor of sesquiterpenes, is derived from the MVA pathway (Newman and Chappell, 1999). *Arabidopsis* genome contains two HMG-CoA reductase (HMGR) genes, *HMGR1* and *HMGR2*, which catalyze the formation of mevalonate, and two farnesyl diphosphate synthase (FPS) genes, *FPS1* and *FPS2* (Enjuto et al., 1994; Cunillera et al., 2000). Analysis with qRT-PCR showed that none of these MVA pathway genes were obviously affected by changed levels of miR156 (Figure 2B), suggesting a possibility that the downstream *TPS* genes could serve as targets of SPLs.

As reported, *TPS11* and *TPS21* are predominantly expressed in inflorescence, with (+)-thujopsene and (*E*)- β -caryophyllene as their major products, respectively (Tholl et al., 2005). Consistent with the great variation of (*E*)- β -caryophyllene emission (Figure 2A), the *TPS21* expression level was increased by more than two-fold in *Pro35S:MIM156* but drastically reduced in *Pro35S:MIR156* inflorescences (Figure 2C). For *TPS11*, neither its expression nor the product emission was significantly affected by miR156 (Figure 2A and 2C).

We then extended the analysis to other *TPS* genes. We found that the expression levels of *TPS14* and *TPS24* were also affected by miR156-SPLs, but the effect was not clear for *TPS03* and two other putative *TPS* genes, *TPS17* and *TPS18* (Supplementary Figure 2 online), whose function has not been identified. Together, these data indicate that miR156 affect a subset of *TPS* genes, with a profound effect on *TPS21* and its product, which dominates *Arabidopsis* floral volatiles. Notably, among the *TPS* genes examined, *TPS21* also exhibited the most clear age-dependent temporal expression pattern in inflorescence (Figure 1A-1E).

Correlated Expression Patterns of *TPS21* and *SPL9*

In *A. thaliana* 11 of the 17 *SPL* genes are targeted by miR156 and they can be divided into four clades: *SPL3/4/5*, *SPL9/15*, *SPL2/10/11* and *SPL6/13A/B* (Guo et al., 2008). We then detected the *TPS21* expression level in transgenic plants expressing the miR156-resistant form of *SPL* genes (*rSPLs*), *Pro35S:rSPL3*, *ProSPL9:rSPL9*, and *Pro35S:rSPL10*. Compared to the wild-type, *TPS21* expression was elevated in all transgenic lines, although to different degrees (Figure 3A). Among the three tested *SPLs*, *SPL9* was most effective in promoting *TPS21* expression, which was then chosen for further investigation.

To dissect the molecular link between *TPS21* and the miR156-targeted *SPLs*, represented here by *SPL9*, we first compared their spatial expression patterns in both vegetative and reproductive organs. We found that *SPL9* had the highest level of transcripts in inflorescence and a moderate level in stem and silique; similarly, *TPS21* transcripts were most abundant in inflorescence (Figure 3B). As reported previously, in stem *SPL9* is expressed in an acropetal manner, with the highest level at the top and the lowest in the junction between stem and rosette leaves (Yu et al., 2010; Gou et al., 2011). *TPS21* exhibited the same expression pattern as *SPL9* with a gradual increase of transcript abundance along the stem from the bottom up (Figure 3C).

To further examine the regulation of *TPS21* expression by *SPLs*, we employed the transgenic plants that expressed a dexamethasone (DEX)-inducible form of *rSPL9* (*ProSPL9:rSPL9-GR*), or an ethanol-inducible miR156 (*ProAlcA:MIR156*). Application of dexamethasone (DEX) to the *ProSPL9:rSPL9-GR* plants doubled the *TPS21* expression (Figure 3D). On the contrary, when *ProAlcA:MIR156* plants were treated with 5% ethanol, in which transient over-production of miR156 would cause the decline of the *SPL* levels, *TPS21* expression was decreased by 50% (Figure 3D). Next, we introduced a *GUS* reporter gene driven by the *TPS21* promoter into *Pro35S:MIM156* or *Pro35S:MIR156* plants, respectively. *GUS* staining was pronouncedly enhanced in *Pro35S:MIM156* but barely visible in *Pro35S:MIR156* inflorescences, compared to that in the wild-type (Figure 3E-3G), suggesting that the *SPLs* might target *TPS21* promoter *in vivo*.

SPL9 Directly Binds to and Activates *TPS21* Promoter

GTAC motif has been identified as the core binding site of SPLs (Klein et al., 1996; Birkenbihl et al., 2005; Kropat et al., 2005; Liang et al., 2008). Within a 1376-bp fragment of *TPS21* gene upstream to the translation start codon, there are eight GTAC motifs (Figure 4A). To assess whether SPL9 could bind to these *cis*-regulatory motifs, an electrophoretic mobility shift assay (EMSA) was performed using a recombinant HIS-SPL9 protein. The 1376-bp fragment was divided into three segments, designated as *cis*1, *cis*2 and *cis*3, with a ~70-bp overlapping between each other (Figure 4A). SPL9 showed a strong binding capacity to *cis*1, a moderate binding to *cis*3, and a weak, if any, binding to *cis*2 (Figure 4B). The specificity of this binding was confirmed in a competition assay with excess amounts of unlabeled probes of *cis*1 and *cis*3, respectively (Figure 4B). One of the possible explanations for the discrepancy among these *cis*-elements is that the adjunct nucleotides around the “GTAC” motif could influence the SPL-DNA interaction.

We then investigated the SPL9 binding capability to *TPS21* promoter *in vivo*. Chromatin immunoprecipitation (ChIP) assay was performed using the *ProSPL9:GFP-rSPL9* transgenic plants, in which *rSPL9* was in-frame fused to the green fluorescent protein (GFP) coding region. Four genomic fragments spanning all the eight GTAC motifs, namely I, II, III and IV (Figure 4A), were examined, among which fragments I, II and IV, but not III, were apparently enriched to varying degrees after pull-down with an anti-GFP antibody (Figure 4C). In accordance to the EMSA results, the affinity between SPL9 and *cis*1 (consisting of fragment I and II) was the tightest, and *cis*3 (consisting of fragment IV) came the second. Taken together, these data indicate that SPL9 binds directly to the promoter of *TPS21* both *in vitro* and *in vivo*.

To see if the SPL binding motifs were required for *TPS21* promoter activity, we constructed five mutant versions of *TPS21* promoter: in *mu*1 the six upstream GTAC motifs were deleted, in *mu*2 and *mu*3 the respective GTAC motif was changed to AAGA, *mu*2+3 was a double mutant containing both *cis*2 and *cis*3 mutations, and

finally the upstream six motifs in *mu2+3* were deleted to form *mu4* that contained no intact GTAC motif at all (Figure 5A).

The intact (*INT*) and different mutant versions of the *TPS21* promoter were fused to the firefly luciferase (LUC) reporter gene, respectively, and the promoter activities were then detected by using a transient dual-LUC assay system in *Nicotiana benthamiana* (Liu et al., 2008).

Compared to mock control, the LUC activity of *INT* was greatly increased when co-transformed with *Pro35S:rSPL9* (Figure 5B), indicating that SPL9 activated *TPS21* expression. The *mu1* and *mu4* mutations seriously impaired, and *mu3* and *mu2+3* compromised this activation to a different extent, whereas the *mu2* mutation did not exhibit any effect (Figure 5B). Similar results were obtained with transgenic plants harboring the *GUS* reporter gene driven by the *TPS21* promoter and its mutant versions. GUS-staining showed a moderate decrease in inflorescence for the *mu2+3* promoter, and a great reduction for the *mu1* and *mu4* promoters (Figure 5C-5F). Thus data from both transient dual-LUC and stable transformation GUS assays demonstrate that the GTAC motifs, particularly those located in *cis1*, are responsible for transcriptional activation of *TPS21* promoter by SPL9.

As *mu2+3* and *mu1* disrupted *TPS21* promoter activities to a different extent, we introduced them into *Pro35S:MIM156* and *Pro35S:MIR156* plants, respectively, by cross. GUS-staining of the inflorescence showed that the *mu2+3* promoter activity was enhanced in *Pro35S:MIM156* but attenuated in *Pro35S:MIR156* backgrounds (Figure 5G and 5H), suggesting that the *cis*-elements outside the region of *mu2+3* (*cis2* and *cis3*) operated well for SPL-mediated *TPS21* promoter activation, which was further supported by the fact that the GUS-staining of *mu1* remained undetectable even in the *Pro35S:MIM156* background (Figure 5I and 5J). Therefore the six upstream GTAC motifs in *cis1* are crucial for transcriptional activation of *TPS21* by SPL9.

SPLs Regulate Sesquiterpene Abundance

As SPLs, in particular SPL9, positively regulate *TPS21* transcription, we determined the amount of sesquiterpene components released from inflorescences of the transgenic plants with different levels of SPL9. The *ProSPL9:rSPL9* plants exhibited a similar phenotype as *Pro35S:MIM156* with skipped juvenile phase and earlier flowering, whereas the *spl9* homozygous mutant (T-DNA insertion, SAIL_150_B05) showed a slight delay in flowering and an increase in branching. The transcript level of *TPS21* was elevated to ~2.5-fold in *ProSPL9:rSPL9* and dropped to less than 50% in *spl9*, respectively, whereas the *TPS11* expression was largely unaffected (Supplementary Figure 3A online). Consistent with gene expression, (*E*)- β -caryophyllene content was elevated by 76% in the *ProSPL9:rSPL9* but halved in the *spl9* mutant emissions, compared to the wild-type. The amount of *TPS11* products emitted, represented by (+)-thujopsene, was much less affected than (*E*)- β -caryophyllene and remained to be low for both transgenic lines (Supplementary Figure 3B online). Clearly, SPL9 plays an important role in regulating *TPS21* gene expression and, consequently, the biosynthesis and emission of (*E*)- β -caryophyllene. Since the *TPS21* expression level was decreased to a greater degree in *Pro35S:MIR156* than in *spl9* (Figure 2C and Supplementary Figure 3A online), and SPL3 and SPL10 also promoted *TPS21* expression (Figure 3A), these miR156-controlled SPLs may function additively in regulating *TPS21* expression.

SPLs Regulate *TPS21* Expression in Parallel with MYC2

Our previous investigation demonstrated that MYC2 directly up-regulates *TPS21* and *TPS11* expression in *Arabidopsis*, and the amount of (*E*)- β -caryophyllene released from inflorescences of two T-DNA insertion mutants of MYC2, *myc2-1* and *myc2-2*, was decreased to about one half of the wild-type (Hong et al., 2012). To see whether MYC2 was involved in SPL-mediated activation of *TPS21*, a mutant *ProTPS21:GUS* harboring an invalid MYC2 binding site (*ProTPS21 E-box mu2:GUS*) in the wild-type and an original *ProTPS21:GUS* in the *myc2-2* mutant plants were transformed with *Pro35S:MIM156* and *Pro35S:MIR156*, respectively. The GUS staining in inflorescence was faint when the second MYC2 binding motif in *TPS21* promoter was

mutated or in the *myc2-2* mutant background, as previously reported (Hong et al., 2012), but *Pro35S:MIM156* and *Pro35S:MIR156* still up- or down-regulated the *TPS21* promoter activity, respectively (Figure 6). These data suggest that both SPLs and MYC2 are involved in regulating *TPS21* expression in inflorescence, and they function independently.

Patchouli Oil Content and *PatPTS* Expression Mount with Age

Patchouli accumulates considerable essential oil, which consists of over 24 different sesquiterpenes instead of a blend of diversified compounds (Buré and Sellier, 2004). The major constituent (-)-patchoulol takes ~35% of the total amount (Deguerry et al., 2006).

We analyzed the patchouli oil contents in leaves collected at different stages of growth. GC-MS showed that in leaf extracts patchoulol content increased along with plant aging (Figure 7A). Patchoulol was undetectable in juvenile plants (one- and two-week old) and became evident in one-month old plants though its level was still low. Since then the amount of patchoulol elevated markedly, and reached to ~12-fold higher in one-year compared to that of the one-month old plant (Figure 7A). Consistently, expression level of *PatPTS* was rising drastically with plant aging (Figure 7B). Although the patchouli oil content in young leaves could be underestimated due to a higher water content, the more than ten-fold increase of the oil amount in the one-year old leaf and the temporal pattern of *PatPTS* gene expression clearly suggest a link between patchoulol accumulation and age-regulated *TPS* gene expression.

SPLs Regulate Sesquiterpene Biosynthesis in Patchouli

Given the facts that in *Arabidopsis* SPL9 directly regulates *TPS21* expression and biosynthesis of the predominant sesquiterpene component (*E*)- β -caryophyllene, we asked if SPLs also regulated sesquiterpene biosynthesis in patchouli. Because over-expressing *rSPL9* (*Pro35S:rSPL9*) could cause plants sterile (Wang et al., 2008), *SPL10* (*Pro35S:rSPL10*) was chosen for patchouli transformation.

Analysis of the ten-month old plant showed that the *PatPTS* transcript level was increased up to 5-fold in *Pro35S:rSPL10* leaves, but decreased to a very low level in *Pro35S:MIR156*, compared to the control (Figure 8A). Consistently, *PatPTS* products were substantially elevated in *Pro35S:rSPL10* leaf extracts (Table 1). Content of the major product, (-)-patchoulol, was elevated by 20% in *Pro35S:rSPL10*; by striking contrast, it was below the detectable level in *Pro35S:MIR156* (Figure 8B and 8C). We then extended qRT-PCR analysis to all five sesquiterpene synthase genes identified from patchouli (Deguerry et al., 2006), of which *PatPTS* showed a much higher expression level than others in leaf, and *PatTpsBF2* expression seemed also affected by miR156 and rSPL10 (Supplementary Figure 4 online). These data indicate that *PatPTS*, the predominant sesquiterpene synthase gene responsible for patchouli oil biosynthesis, is under the control of miR156-targeted SPLs.

Leaves of these transgenic lines were then regenerated to produce re-juvnescent plants by tissue culture. In leaves of the one-month old regenerated lines of *Pro35S:rSPL10*, *PatPTS* expression was elevated obviously, as well as the (-)-patchoulol content (Figure 8D and 8E). *PatPTS* expression and (-)-patchoulol content were sharply decreased in *Pro35S:MIR156* (Figure 8D and 8E). Together, these results demonstrate that the miR156-targeted SPLs up-regulate the *PatPTS* expression and patchouli oil biosynthesis in the perennial herb patchouli.

DISCUSSION

Secondary metabolites contribute greatly to plant adaptation to unpredictable environments and successful reproduction (Pichersky and Gershenzon, 2002; Falara and Pichersky, 2012). These small organical compounds can be produced instantly in plant cells in response to external stimuli, during which phytohormones, such as JA, are the major mediators in transmitting the induction signals (Memelink et al., 2001; Balbi and Devoto, 2008). On the other hand, secondary metabolites are often synthesized and stored in specific structures or tissues of defined organs, and the

metabolite profile varies along with plant growth and development (Bouwmeester et al., 1998; Turner et al., 2000; Chen et al., 2003; Dudareva et al., 2003; Tholl and Lee, 2011); in this regard, plant secondary metabolism must be linked to development.

We have demonstrated that, in *A. thaliana*, the miR156-targeted SPLs show a direct and strong regulation on *TPS21*, which is responsible for the biosynthesis of (*E*)- β -caryophyllene, a predominant component of the sesquiterpene volatiles released from flowers. We also found that miR156-SPLs play a similar role in patchouli in promoting the expression of *PatPTS* and the production of patchouli oil. *PatPTS* is a predominant sesquiterpene synthase gene of patchouli regarding its expression level in leaf and critical role in biosynthesis of patchouli oil. Thus in both species, the sesquiterpene synthase selected by miR156-targeted SPLs become the dominant enzyme in producing sesquiterpenes, at least at a defined developmental stage. We propose that the miR156-controlled SPLs play an important role in determining the formation and composition of terpenoids in each plant species by recruiting and substantially up-regulating specific members of the *TPS* gene family, thus contributing to the chemical identity of the plant.

Arabidopsis is taken as a self-pollinated plant, however, distinct genomic recombinations exist among accessions of different origin (Stahl et al., 1999; Kuittinen and Aguade, 2000; Haubold et al., 2002). The observation that flowers are occasionally visited by insects in the wild (Hoffmann et al., 2003), which may cause pollen transfer between populations, provides a good explanation to the genomic recombination. The emitted terpenes may serve as an olfactory cue to attract pollinators. As the rates of cross pollination are low, floral volatiles may have other functions. For example, the major sesquiterpene of *Arabidopsis* floral volatiles, (*E*)- β -caryophyllene, has the activity of directly inhibiting bacterial growth on the sigma (Huang et al., 2012). Though to date there have been no comprehensive investigations on the role of patchoulol in plant biotic or abiotic defense, it could serve as anti-microbe regents and insect deterrents (Wu et al., 2006; Zaim et al., 2013). In any case, the involvement of miR156-SPLs in regulating the developmental

biosynthesis of terpenoid confers a lot to the successful reproduction of the plant population (Wu et al., 2006; Huang et al., 2012; Zaim et al., 2013).

Besides *TPS21*, a subset of *TPS* genes of *Arabidopsis* are progressively up-regulated in inflorescence with plant age. A plausible explanation is that, in addition to miR156 targeted SPLs, other transcription factors also participate in regulation of sesquiterpene biosynthesis. As we illustrated for *Arabidopsis*, both the JA signaling pathway transcription factor MYC2 and the miR156-targeted SPLs are involved in regulating terpene biosynthesis in flower, and they act on *TPS21* gene expression in parallel. In *A. annua*, both AaWRKY1 and AaERFs promote artemisinin biosynthesis by activating *ADS* expression (Ma et al., 2009; Yu et al., 2012b). And in tobacco, NtMYC2 controls nicotine biosynthesis by directly targeting *putrescine N-methyltransferase 2 (PMT2)* and *quinolinate phosphoribosyltransferase 2 (QPT2)* genes, or indirectly by up-regulating the *NIC2*-locus *ERF* genes which then activate *PMT2* and *QPT2* expression (Shoji and Hashimoto, 2011; Zhang et al., 2012). Thus it is a common feature that multiple transcription factors control a single specialized metabolic pathway. Furthermore, it becomes an emerging phenomenon in plant that genes involved in the biosynthesis of a specific group of secondary metabolites tend to be clustered, which provides a cost-effective way for concerted regulation (Frey et al., 1997; Qi et al., 2004; Wilderman et al., 2004; Swaminathan et al., 2009; Falara et al., 2011; De Luca et al., 2012; Winzer et al., 2012; Mugford et al., 2013).

We previously reported that DELLA proteins, the negative regulator of GA signaling pathway, interact with MYC2 and repress its activity on regulating *TPS21* and *TPS11* expression in flowers (Hong et al., 2012). A more recent report demonstrated that DELLAs interact with SPLs and this interaction disrupts the SPL-mediated activation on MADS box genes at the shoot apex and in turn delays flowering under both short-day and long-day conditions (Porri et al., 2012; Yu et al., 2012a). Although we found that the SPL regulation of *TPS21* expression is independent of MYC2, this does not preclude a possible interplay between DELLA, SPL and MYC2, which may integrate both inner clues of plant development and

external stimuli of environmental stresses into orchestration of plant secondary metabolism.

It has been reported in many plant species of different families that the terpenoid contents change during plant development. In addition to the species of Lamiaceae introduced above, lemongrass (*Cymbopogon flexuosus*) of the grass family (Poaceae) also shows age-dependent accumulation of terpenes (Singh et al., 1989; Lewinsohn et al., 1998). Furthermore, sesquiterpene levels were found to increase gradually along the internodes in an acropetal manner in *Solanum habrochaites* of the Solanaceae family (Besser et al., 2009). In plant kingdom miR156 is evolutionally conserved; the miR156 level declines and the target SPLs accumulate with plant age (Axtell and Bartel, 2005; Wang et al., 2009; Wu et al., 2009; Huijser and Schmid, 2011). It would be greatly interesting to examine if the miR156-SPLs operate in a wide range of plant taxa in connecting plant development and aging to terpene biosynthesis. As demonstrated herein, over-expression of a *SPL* gene in patchouli not only increased patchouli oil content but also accelerated plant growth and the transition from juvenile to adult vegetative phases, this provides a basis to design novel strategies to boost the essential oil yield in a shortened period of plant growth.

METHODS

Plant Materials and Constructs

Arabidopsis thaliana (ecotype, Columbia-0) and *Nicotiana benthamiana* plants were grown at 22 °C in long days (16 h light/8 h dark), and patchouli (*Pogostemon cablin*) in a similar condition at 26 °C. The cultivar of patchouli was from Firmenich.

The 1376-bp *Arabidopsis TPS21* promoter fragment was amplified using PrimeSTAR DNA Polymerase (Takara) and mutated by two-round PCR. The intact *TPS21* promoter was referred as *INT* which contained 8 GTAC motifs. For promoter mutants shown in Figure 5A, *mu1* was a deletion of six upstream GTAC motifs in cis1; *mu2* and *mu3* were the single mutant of the GTAC motif either cis2 or cis3, which

was changed to AAGA, respectively; *mu2+3* is the combination of *mu2* and *mu3* mutations; and *mu4* contains all mutations. For β -glucuronidase (GUS) assay, these constructs were fused to *GUS* reporter gene, introduced into *Arabidopsis*, and the GUS staining was performed as described (Jefferson et al., 1987). For Dual-LUC assay using *N. benthamiana* leaves, the promoters were fused to firefly luciferase (*LUC*) reporter gene. Primers used in this investigation are listed in Supplementary Table 1 online.

Plant Treatments

For transient up-regulation of *SPL9*, the 2-week old *ProSPL9:rSPL9-GR Arabidopsis* plants were sprayed with either DEX or DMSO (control), both at 10 μ M. The rosette leaves were collected after a 5-hour treatment. For transient down-regulation of miR156-targeted *SPL* genes, the 4-week old *ProAlcA:MIR156* bolting plants of *Arabidopsis* were sprayed with 5% ethanol or water (control), and the inflorescences were harvested after 6 hours.

Patchouli Transformation

Plants of patchouli, line L002, was transformed. *Agrobacterium tumefaciens* EHA105 strains harboring *Pro35S:MIR156*, *Pro35S:rSPL10* and *Empty Vector* were cultured overnight and resuspended in infection medium (1/2 MS, 45 g/L sucrose, 200 mM acetosyringone). Leaf dishes were then infected for 5 min and incubated at 24°C for 2 days on co-culture medium (MS, 0.25 mg/L 6-benzyl aminopurine, 0.25 mg/L kinetin, 0.25 mg/L trans-zeatin, 0.25 mg/L indole-3-butyric acid, 100 mM acetosyringone), followed by incubation on selective differentiation medium (MS, 0.25 mg/L 6-benzyl aminopurine, 0.25 mg/L kinetin, 0.25 mg/L trans-zeatin, 0.25 mg/L indole-3-butyric acid, 500 mg/L carbenicillin, 100 mg/L kanamycin). Four weeks later, the greenish calli with adventitious shoot buds were transferred to selective elongation medium (MS, 0.1 mg/L 6-benzyl aminopurine, 300 mg/L carbenicillin, 100 mg/L kanamycin). Kanamycin resistant shoots were rooted on the MS basal medium containing 200 mg/L carbenicillin and 50 mg/L kanamycin.

Expression Analysis

Total RNA was extracted from plant materials with Trizol reagent (Invitrogen). One μg of total RNA, after treatment with DNase I, was used for cDNA synthesis with oligo(dT) primers and M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Invitrogen). Quantitative real-time RT-PCR was performed with SYBR-Green PCR Mastermix (Takara), and amplification was real-time monitored on a Mastercycler RealPlex² (Eppendorf). As internal reference β -TUBULIN2 was used for *Arabidopsis* and *Pat18S* (18S ribosomal RNA gene) for patchouli.

Volatile Analysis

The analysis was performed as previously described (Hong et al., 2012). Briefly, ~0.5 g inflorescence samples were detached from the 5-week old *Arabidopsis*, which were incubated in μ -CTE (Micro-Chamber/Thermal Extractor) at 30 °C for 1 h; the volatiles were collected by Thermal Desorption Autosampler (Markes) with the Tenax TA (porous polymer) sorbent in a nitrogen gas flow. The adsorbed volatiles were desorbed and injected by Thermal Desorber (TD100, Markes), followed by a GC-MS (HP 6890/5973GC-MSD) analysis with Nonyl acetate (10 ng, Aldrich) as internal standard, the GC-MS analysis was performed as described (Chen et al., 2003).

Patchouli Oil Extraction and GC-MS Analysis

Nine discs from the sequential 3 fully expanded leaves (from the top) were picked with hole punch ($\phi=1\text{cm}$). After weighing the materials were ground into powder with liquid nitrogen and re-suspended with 1.5 ml extraction buffer (hexane: ethyl acetate = 85:15) which contained 20 ng/ μl nonyl acetate as internal standard. The suspension was further sonicated for 20 min and then centrifuged at 5,000 g for 5 minutes at room temperature. The supernatant was dehydrated with anhydrous Na_2SO_4 and used for GC-MS analysis. The column temperatures were programmed as follow: 80 °C for 2 min, raised to 120 °C at 3 °C/min and hold for 10 min, then raised to 170 °C at 5 °C/min and hold for 10 min, finally raised to 250 °C at 20 °C/min and hold for 2 min. The injector temperature was 250 °C. Helium was the carrier gas at 1.5 ml/min.

Electrophoretic Mobility Shift Assay (EMSA)

The ORF of *SPL9* was in frame fused to the HIS-tag of the expression vector pET32a (Novagen), and the recombinant HIS-SPL9 proteins were affinity purified following the manual. For EMSA, the *TPS21* promoter segments, namely cis1, cis2 and cis3, were labeled with Cy5 on both ends. The DNA fragment and the purified proteins were incubated at 25 °C for 30 min in the buffer containing 1 mg/ml poly (dI-dC) (Roche, USA), 20 mM Tris-base (pH 7.5), 1 mM dithiothreitol (DTT), 10 mM MgCl₂, 0.5 mg/ml calf BSA and 5% glycerol and then separated by 5% native polyacrylamide gelelectrophoresis in 0.5×TBE buffer at 10 V/cm and 4°C. The fluorescence was detected with FUJIFILM FLA-9000 image scanner.

Chromatin Immunoprecipitation (ChIP) Assay

About 5 g flower buds of 4-week old *ProSPL9:GFP-rSPL9* and the wild-type plants were harvested, respectively. After fixation, the materials were washed and ground into powder in liquid nitrogen and resuspended in extraction buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 5 mM mercaptoethanol, 0.1 mM PMSF, and 1× protease inhibitor) and lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate sodium, and 0.1% SDS) in turns, followed by sonification (output 3, 6×10 s). One third of the crude chromatin extracts was saved as input control and the other two thirds were treated with anti-GFP or anti-HA (Abmart) antibodies, separately. After precipitation the resulting DNA samples were purified with a PCR purification kit (Qiagen). The abundance of the DNA fragments was analyzed by qRT-PCR with *β-TUBULIN2* promoter as a reference, as described (Yu et al., 2010). Relative enrichment of each fragment was calculated first by normalizing the value for anti-GFP against the value for anti-HA and then compared the result of *ProSPL9:GFP-rSPL9* to that of the wild-type plants.

Dual-luciferase (Dual-LUC) Assay of Transient Regulation

The assay was performed following a published protocol (Liu et al., 2008). In brief, the wild-type and the mutant versions of *TPS21* promoter were inserted into

pGreen-LUC plasmid driving the firefly luciferase reporter gene with the Renilla (REN) luciferase controlled by the constitutive 35S promoter on the same plasmid as a reference to normalize infection efficiency. The constructs were then transferred into *Agrobacterium* (strain GV3101) with the helper plasmid, *pSoup-P19*, which also encodes a repressor of co-suppression. The transformed *Agrobacterium* cells were mixed with the *Agrobacterium* strains containing the effector *Pro35S:rSPL9* or the *Empty Vector* in a ratio of 1:4.

Transient transformation was conducted by infiltration of the *Agrobacterium* mixture into the abaxial side of *N. benthamiana* leaves using a syringe. After culturing for 3d, the infected area was collected for total protein extraction. The supernatant of the total protein was reacted with commercial Dual-LUC reaction (DLR) reagents (Promega, E1910), and the fluorescent values of LUC and REN were detected with a luminometer (BG-1, GEM Biomedical Inc), successively. The value of LUC was first compared to that of REN and then the ratio (LUC/REN) co-transformed with *Pro35S:rSPL9* was normalized against the ratio co-transformed with *Empty Vector*.

Accession Numbers

Arabidopsis Genome Initiative gene identifiers are as follows:

SPL3 (At2g33810), *SPL9* (At2g42200), *SPL10* (At1g27370), *MIR156f* (At5g26147), *TPS03* (At4g16740), *TPS11* (At5g44630), *TPS14* (At1g61680), *TPS17* (At3g14490), *TPS18* (At3g14520), *TPS21* (At5g23960), *TPS24* (At3g25810), *HMGR1* (At1g76490), *HMGR2* (At2g17370), *FPS1* (At4g17190), *FPS2* (At5g47770) and β -*TUBULIN2* (At5g62690). Mutants and transgenic plants of *spl9* (SAIL_150_B05), *myc2-2* (SALK_083483), *Pro35S:MIR156*, *Pro35S:MIM156*, *Pro35S:rSPL3*, *ProSPL9:rSPL9*, *Pro35S:rSPL10*, *ProSPL9:rSPL9-GR*, *ProAlcA:MIR156*, *ProSPL9:GFP-rSPL9* and *ProTPS21mu2:GUS* (renamed as *ProTPS21 E-box mu2:GUS*) were as described previously (Wang et al., 2009; Wu et al., 2009; Hong et al., 2012).

Patchouli genes mentioned in this article can be accessed as follows: *PatTpsA* (AY508726), *PatTpsBF2* (AY508727), *PatTpsCF2* (AY508728), *PatTpsB15* (AY508729), *PatPTS* (AY508730), *Pat18S* (FJ980282).

SUPPLEMENTARY DATA

Supplementary Data are available at Molecular Plant online.

Supplementary Figure 1. GC-MS Chromatogram of Volatile Sesquiterpenes Collected from the *Pro35S:MIM156*, Wild-Type and *Pro35S:MIR156* Inflorescence Emissions.

Supplementary Figure 2. Effects of miR156 on the Expression of *TPS* Genes in Inflorescence.

Supplementary Figure 3. SPL9 Up-regulates *TPS21* Expression and (*E*)- β -caryophyllene Formation.

Supplementary Figure 4. Expression Levels of Five Sesquiterpene Synthase Genes in Leaves of Patchouli.

Supplementary Table 1. Oligonucleotide Primers Used in This Investigation.

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Table 1. Contents (% FW) of Sesquiterpene Constituents in Patchouli Leaf Essential Oil (Patchouli Oil).

Sesquiterpenes	<i>Empty Vector</i>	<i>Pro35S:rSPL10</i>
β -patchoulene	0.020 $\pm$ 0.007	0.028 $\pm$ 0.005 ^b
β -elemene	0.019 $\pm$ 0.007	0.024 $\pm$ 0.005
trans- β -caryophyllene	0.037 $\pm$ 0.019	0.053 $\pm$ 0.011
α -guaiene	0.266 $\pm$ 0.075	0.311 $\pm$ 0.055
α -patchoulene	0.062 $\pm$ 0.021	0.078 $\pm$ 0.014
γ -patchoulene	0.029 $\pm$ 0.009	0.037 $\pm$ 0.007 ^b
guai-4,11-diene	0.050 $\pm$ 0.017	0.062 $\pm$ 0.012
α -bulnesene	0.279 $\pm$ 0.082	0.348 $\pm$ 0.057
(-)-patchoulol	0.314 $\pm$ 0.099	0.429 $\pm$ 0.063 ^b
total	1.032 $\pm$ 0.368	1.369 $\pm$ 0.227 ^b

The plants were transformed with *Empty Vector* (as control) or *Pro35S:rSPL10*. At least 6 plants ten-month old, of each lines were analyzed. The amount of sesquiterpenes was first calculated by comparing with the internal standard and then divided by the fresh weight (FW) of the sample. The nine sesquiterpene compounds listed are all present in the products of PatPTS (Deguerry et al., 2006; Wu et al., 2006). Values are mean $\pm$ SD. Student's *t* test, ^bP < 0.05.

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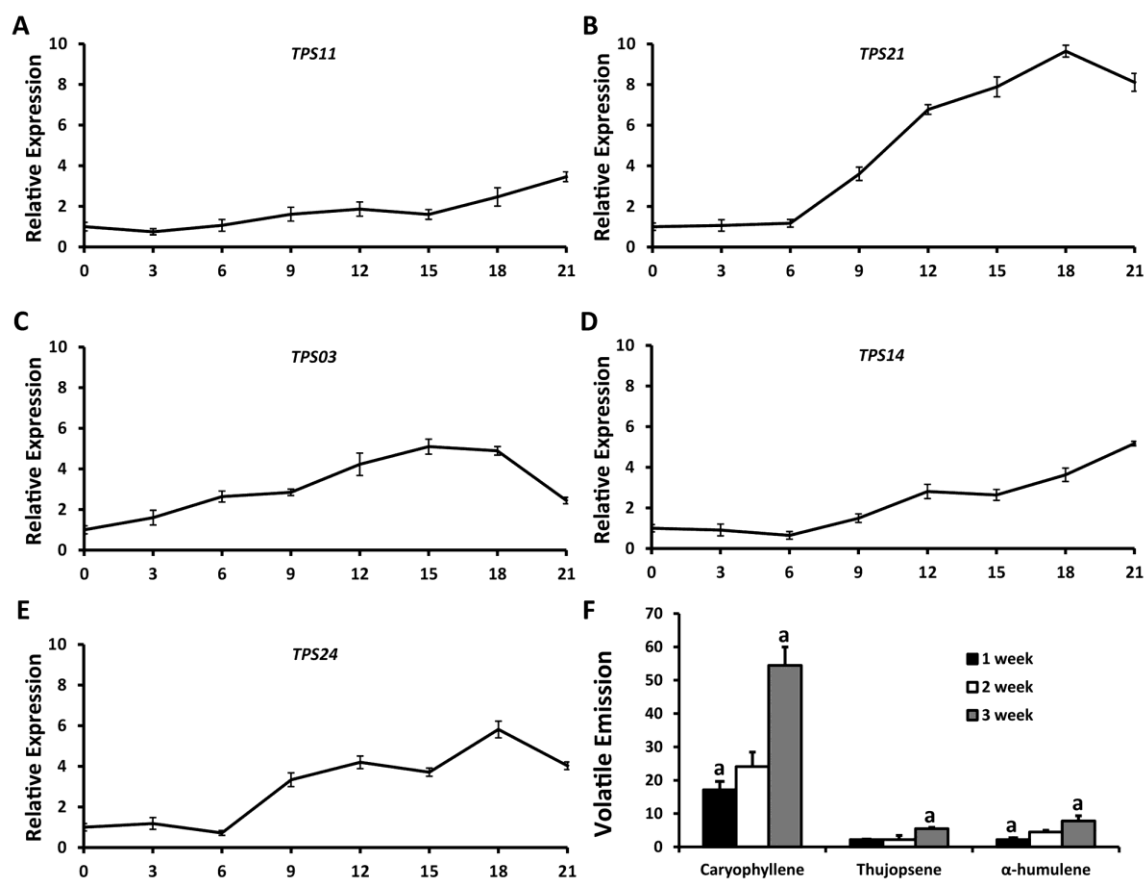


Figure 1. Progressive Up-regulation of *TPS* Genes and Sesquiterpene Emissions.

(A-E) The expression levels of *TPS11* (A), *TPS21* (B), *TPS03* (C), *TPS14* (D) and *TPS24* (E) in inflorescences of different ages (the inflorescences were collected at different days after flowering and the day of bolting was indicated as 0 in X-axis). The level in inflorescences of day 0 was set to 1. Error bars indicate SD of three biological replicates.

(F) Emissions of (*E*)-β-caryophyllene, (+)-thujopsene and α-humulene from the wild-type inflorescences of different ages (nanograms/gram fresh weight and hour [ng/g FW•h]). The inflorescences were collected at different weeks after bolting. Error bars indicate SD of three biological replicates. Student's *t* test, **P* < 0.01. The statistical significance differences between one-week and two-week old inflorescences, and between two-week and three-week old inflorescences, are calculated, respectively.

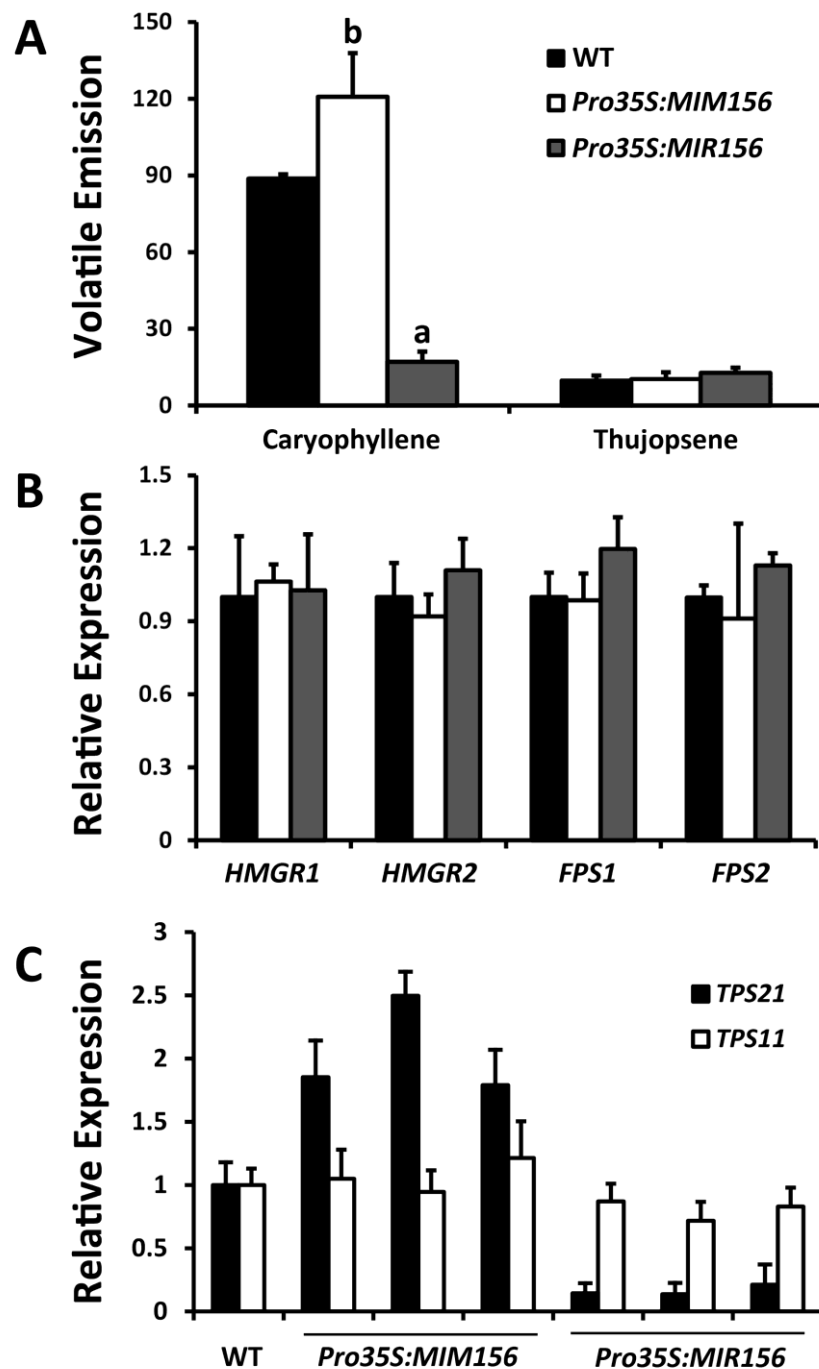


Figure 2. Effects of miR156 on *Arabidopsis* Sesquiterpene Biosynthesis.

(A) Emissions of (*E*)- β -caryophyllene and (+)-thujopsene from the wild-type (WT), *Pro35S:MIM156* and *Pro35S:MIR156* inflorescences (ng/g FW•h) at three weeks after bolting. Error bars indicate SD of three biological replicates. Student's *t* test, ^a*P* < 0.01 and ^b*P* < 0.05.

(B) qRT-PCR analysis of the expression levels of MVA pathway genes of *HMGR1*, *HMGR2*, *FPS1* and *FPS2* in inflorescences. The level in the wild-type was set to 1. Error bars indicate SD of three biological replicates.

(C) qRT-PCR analysis of *TPS21* and *TPS11* expression levels in inflorescences. Error bars indicate SD of three biological replicates.

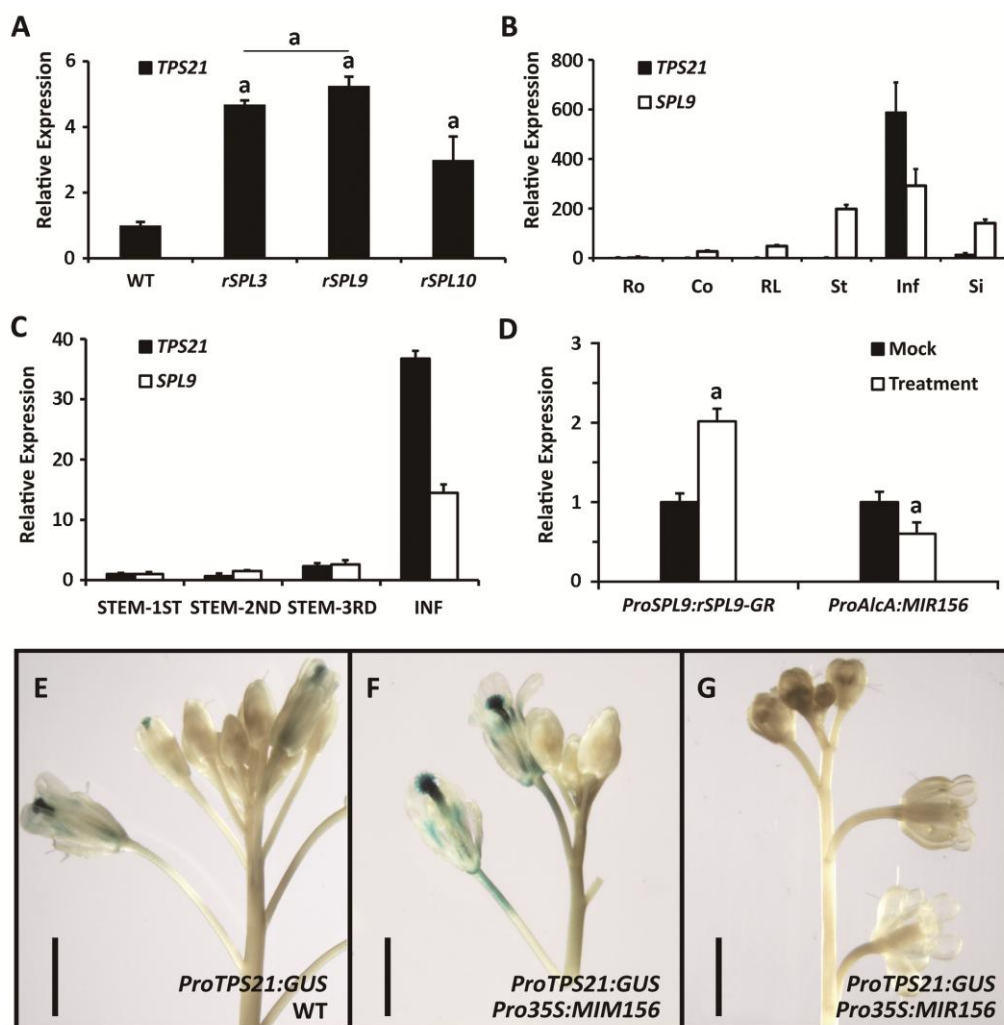


Figure 3. Expression of *TPS21* is Regulated by miR156-targeted SPLs.

(A) Transcript levels of *TPS21* in inflorescences of the wild-type (WT), *Pro35S: rSPL3* (*rSPL3*), *ProSPL9: rSPL9* (*rSPL9*) and *Pro35S: rSPL10* (*rSPL10*) plants, analyzed by qRT-PCR. The level in the wild-type was set to 1. Error bars indicate SD of three biological replicates. Student's t test, ^aP < 0.01. The statistical significance differences between wild-type and different *rSPL* transgenic plants (*rSPL3*, *rSPL9* or *rSPL10*), and between *rSPL9* and *rSPL3*, are calculated respectively.

(B) qRT-PCR analysis of transcript levels of *TPS21* and *SPL9* in root (Ro), cotyledon (Co), rosette leaf (RL), stem (St), inflorescence (Inf) and silique (Si) of *Arabidopsis* as indicated. The levels of *TPS21* and *SPL9* in root were set to 1, respectively. The one-week old seedlings were used for the root and cotyledon analysis, and one-month old plants were used for the rest tissues. Error bars indicate SD of three biological replicates.

(C) Spatial expression patterns of *TPS21* and *SPL9* in main stem and inflorescences of wild-type *Arabidopsis*. 1st to 3rd represent three successive internodes of the main stem from the bottom up. The levels of *TPS21* and *SPL9* in the 1st internode of the main stem were set to 1, respectively. Error bars indicate SD of three biological replicates.

(D) Transient expression of *TPS21* in *ProSPL9: rSPL9-GR* rosette leaves (before bolting) after DEX (left) treatment and in *ProAlcA: MIR156* inflorescences after ethanol (right) treatment. Values from *ProSPL9: rSPL9-GR* plants treated with DMSO and *ProAlcA: MIR156* with water were set to 1 and used as control. Error bars indicate SD of three biological replicates. Student's t test, ^aP < 0.01.

(E) to (G) GUS-staining assay of *TPS21* promoter activity in wild-type **(E)**, *Pro35S: MIM156* **(F)** and *Pro35S: MIR156* **(G)** inflorescences. Bar = 1 cm.

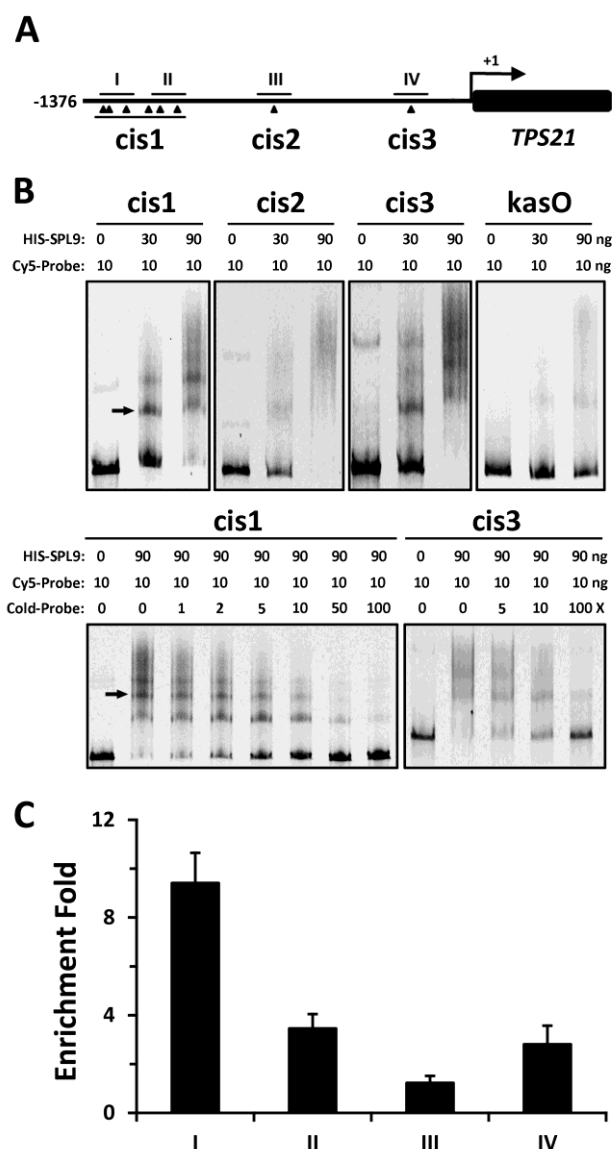


Figure 4. SPL9 Binds to *TPS21* Promoter.

(A) Diagram of *TPS21* promoter region. Solid triangles stand for GTAC elements in *TPS21* promoter region, which was divided into three segments (cis1, cis2 and cis3) with ~70bp nucleotides overlapping between each other, and used in EMSA assay; I to IV represent the DNA fragments used in ChIP analysis (see below). Black box indicates *TPS21* coding region.

(B) EMSA assay of SPL9 binding ability to *TPS21* promoter. Ten ng of Cy5-labeled cis1, cis2 and cis3 fragments were incubated with different concentrations of HIS-SPL9 fusion protein (0, 30 and 90 ng) (upper), the *kasO* promoter fragment of *Streptomyces coelicolor* was used as a negative control. Then the labeled cis1 and cis3 were incubated with 90 ng HIS-SPL9 to compete with different concentrations of cold probes of cis1 (0, 0, 1, 2, 5, 10, 50, 100×) and cis3 (0, 0, 5, 10, 100×), respectively (lower). The specific bands are indicated with a solid arrow.

(C) ChIP enrichments of *TPS21* promoter regions bound by GFP-SPL9. Inflorescences of the four-week old *ProSPL9:GFP-rSPL9* and the wild-type plants were used, DNA fragments were analyzed by qRT-PCR with the β -*TUBULIN2* promoter as a reference. Enrichment of each fragment was referred to the *ProSPL9:GFP-rSPL9* against wild-type plants. Error bars indicate SD of three separate samples.

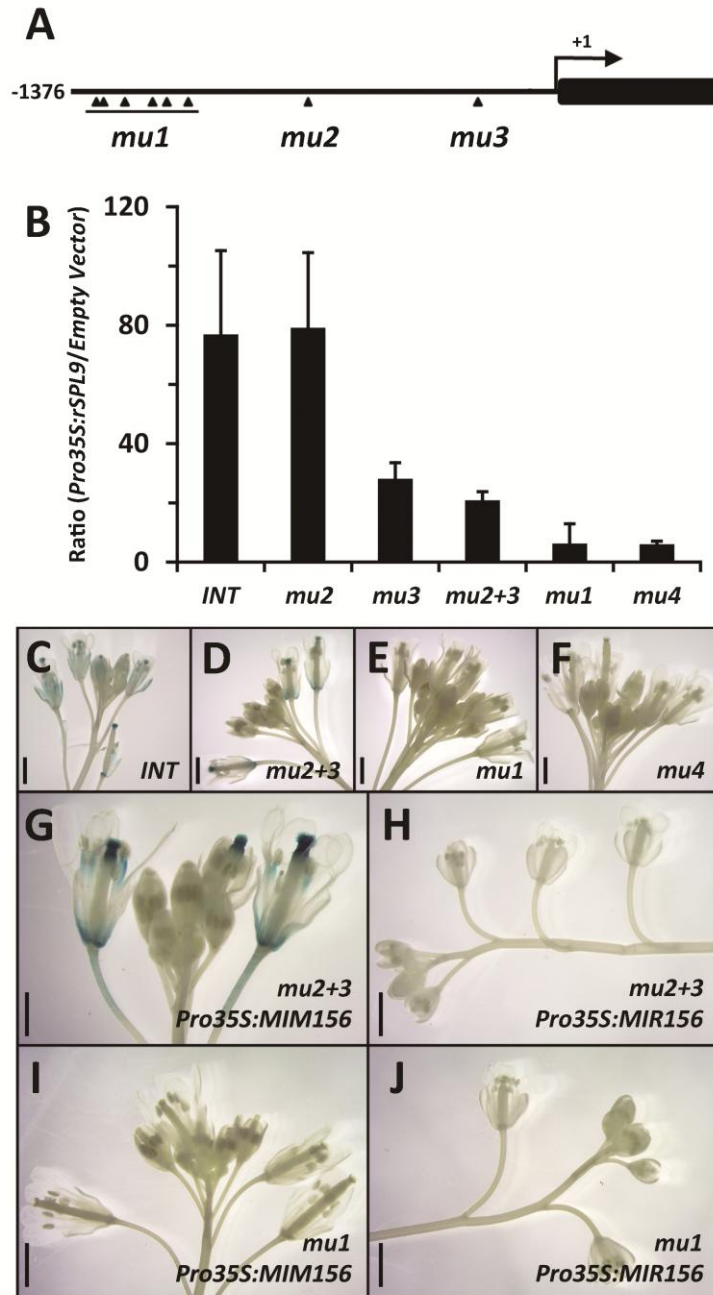


Figure 5. SPL Binding Sites are Required for *TPS21* Promoter Activity.

(A) The eight GTAC boxes in *TPS21* promoter region were deleted or mutated, shown as μ 1, μ 2 and μ 3, the black box indicates reporter genes (*GUS* or *LUC*). For details of promoter mutation, see Methods.

(B) Relative activity of SPL9 on the intact and mutant versions of *TPS21* promoter in transiently transformed *N. benthamiana* leaves. Both the intact (*INT*) and the mutant versions of *TPS21* promoter were fused to *LUC* reporter. The relative *LUC* activities were first normalized to *REN* and then the ratio (*LUC/REN*) of the samples co-transformed with *Pro35S:rSPL9* was compared to that with *Empty Vector*. Error bars indicate SD of three biological replicates.

(C) to (F) *GUS* staining of inflorescences. *GUS* reporter was driven by the intact (*INT*) and mutant (μ 1, μ 2+3, μ 4) *TPS21* promoter respectively. Bar = 2 mm.

(G) to (J) *GUS* staining of inflorescences showing the μ 2+3 and μ 1 promoter activity in *Pro35S:MIM156* (left) and *Pro35S:MIR156* (right) backgrounds. Note that μ 1 totally blocked the activation of *TPS21* promoter by SPLs. Bar = 2 mm.

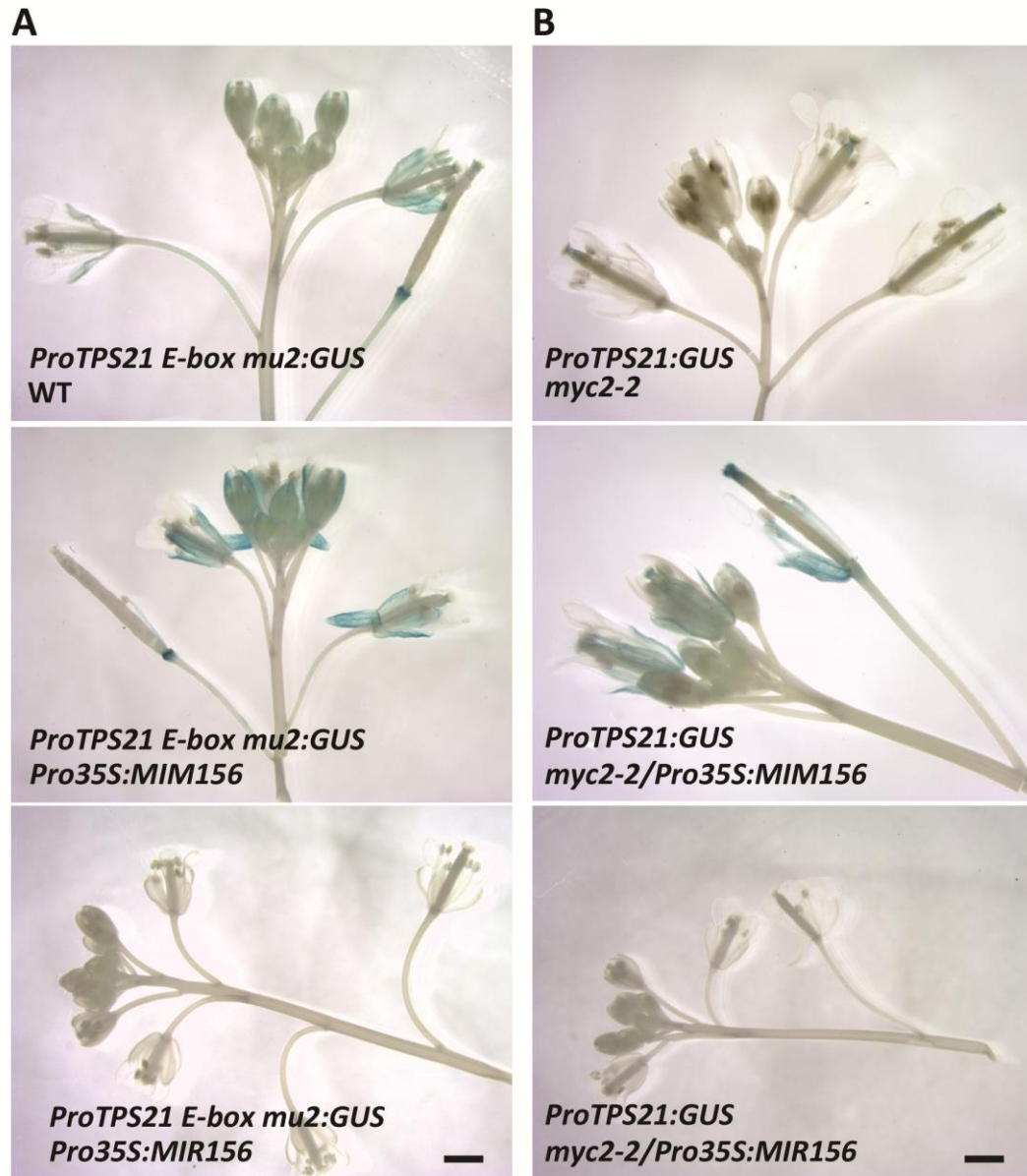


Figure 6. SPLs and MYC2 Regulate *TPS21* Expression in Parallel.
(A) GUS staining showing activity of the mutant *TPS21* promoter (one E-box was mutated, referred as *ProTPS21 E-box mu2:GUS*) in the WT, *Pro35S:MIM156* and *Pro35S:MIR156* backgrounds. Bars = 2 mm.
(B) GUS staining of the intact *TPS21* promoter activity in *myc2-2*, *myc2-2/Pro35S:MIM156* and *myc2-2/Pro35S:MIR156* backgrounds. Bars = 2 mm.

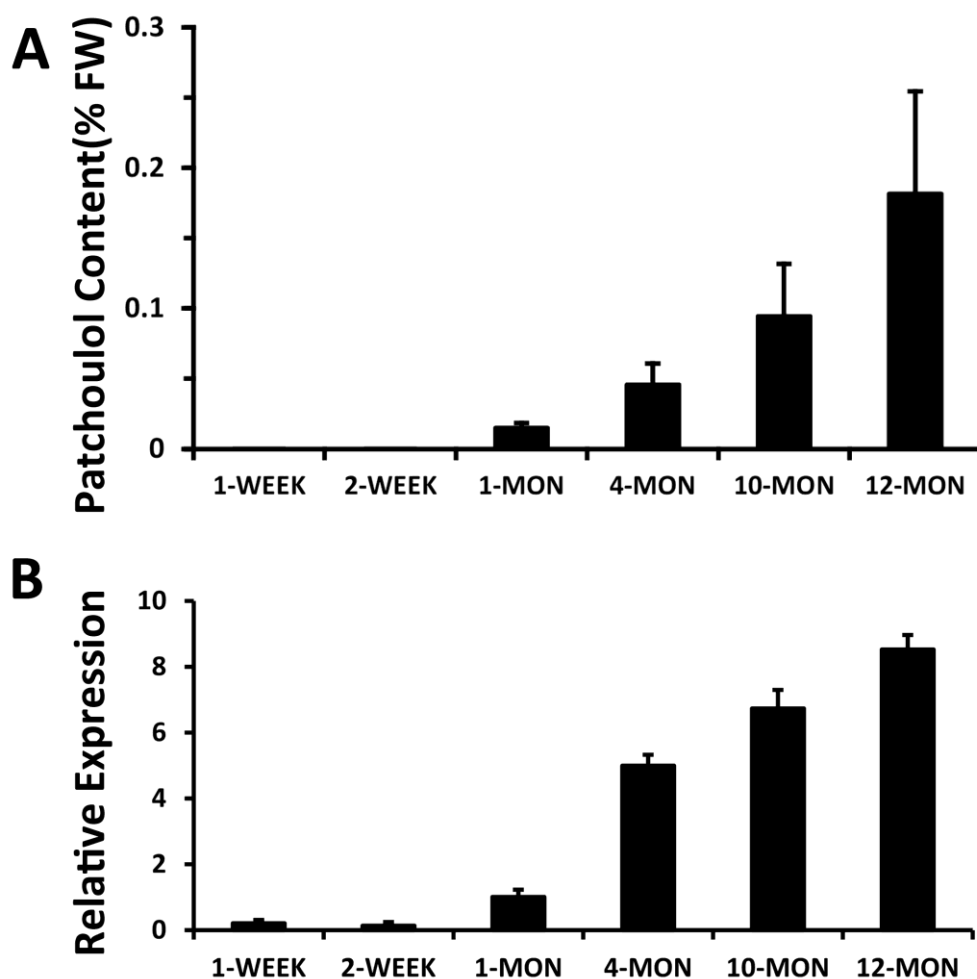


Figure 7. Content of Patchoulol, a Major Constituent of Patchoulol Oil and *PatPTS* Expression in Patchouli Leaf of Different Plant Ages.

(A) Contents of patchoulol in patchouli leaf of different ages as indicated. The amount of (-)-patchoulol was calculated by comparing to the internal standard and then divided by the fresh weight (FW) of the sample. Error bars indicate SD of three biological replicates.

(B) qRT-PCR analysis of *PatPTS* transcript level in leaves. The second fully expanded leaf (from top) was chosen. Error bars indicate SD of three biological replicates.

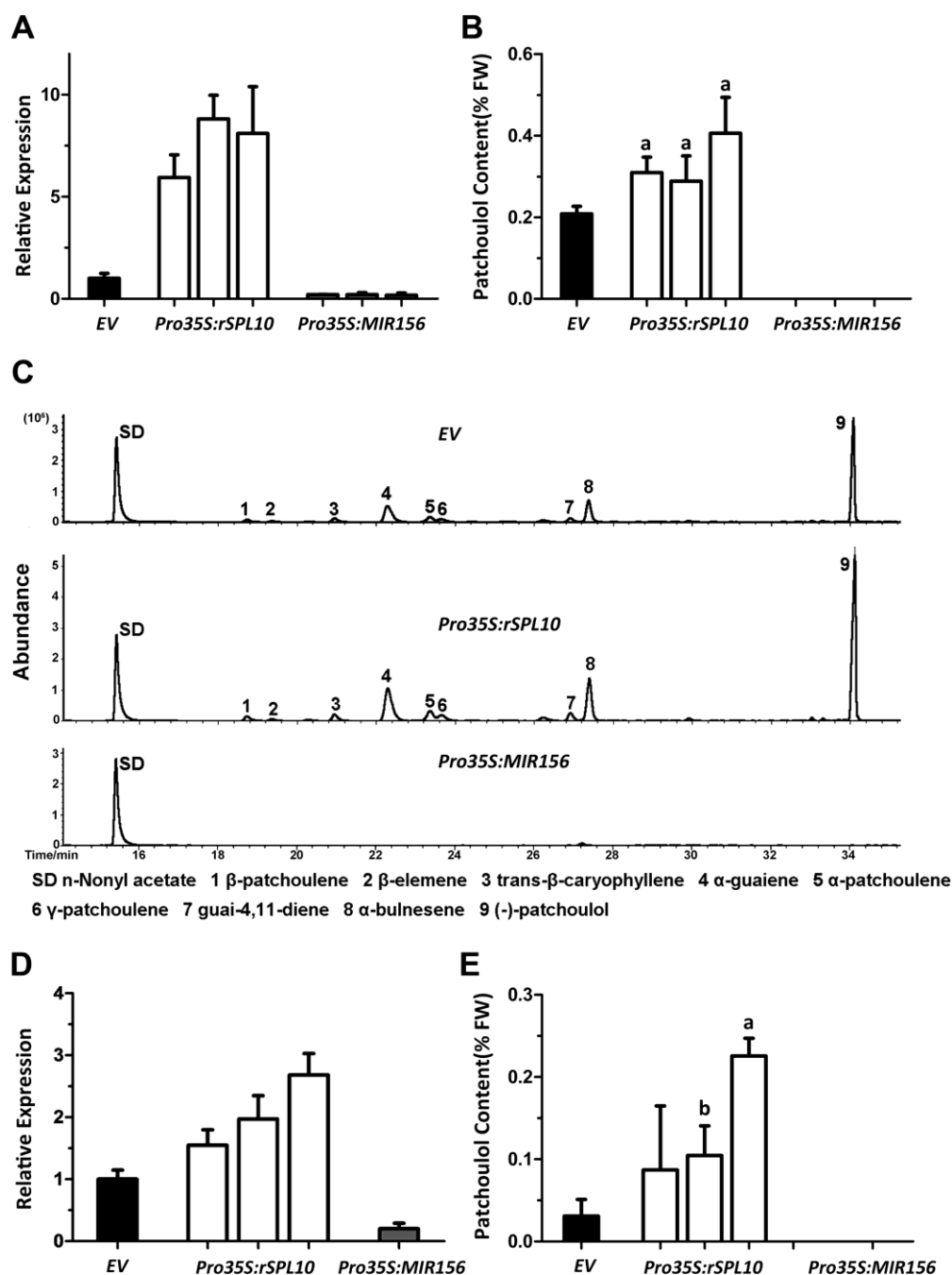


Figure 8. miR156-targeted SPLs Regulate *PatPTS* Expression and Patchoulol Biosynthesis.

(A) Expression level of *PatPTS* in leaves of the ten-month old Empty Vector (EV), *Pro35S:rSPL10* and *Pro35S:MIR156* patchouli plants. The second fully expanded leaf was chosen. Error bars indicate SD of three biological replicates.

(B) Content of (-)-patchoulol in leaves of the ten-month old patchouli plants. The amount of (-)-patchoulol was first calculated by comparing with the internal standard and then divided by the fresh weight (FW) of the sample. Error bars indicate SD of at least three biological replicates. Student's t test, ^aP < 0.01.

(C) GC-MS chromatogram of leaf extracts of the ten-month old patchouli, showing sesquiterpene components. Nonyl acetate was included as an internal standard (SD). Note that sesquiterpenes were undetectable in *Pro35S:MIR156* leaves.

(D) Expression level of *PatPTS* in leaves of the one-month old regenerated patchouli plants. The second fully expanded leaf was analyzed. Error bars indicate SD of three biological replicates.

(E) Contents of (-)-patchoulol in leaf of the one-month old patchouli plants. The amount of (-)-patchoulol was first calculated by comparing with the internal standard and then divided by the fresh weight (FW) of the sample. Error bars indicate SD of three biological replicates. Student's t test, ^aP < 0.01 and ^bP < 0.05.